

Research Paper

Multiple organ gigantism caused by mutation in *VmPPD* gene in blackgram (*Vigna mungo*)

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Seed size is one of the most important traits in leguminous crops. We obtained a recessive mutant of blackgram that had greatly enlarged leaves, stems and seeds. The mutant produced 100% bigger leaves, 50% more biomass and 70% larger seeds though it produced 40% less number of seeds. We designated the mutant as *multiple-organ-gigantism (mog)* and found the *mog* phenotype was due to increase in cell numbers but not in cell size. We also found the *mog* mutant showed a *rippled leaf (rl)* phenotype, which was probably caused by a pleiotropic effect of the mutation. We performed a map-based cloning and successfully identified an 8 bp deletion in the coding sequence of *VmPPD* gene, an orthologue of *Arabidopsis PEAPOD (PPD)* that regulates arrest of cell divisions in meristematic cells. We found no other mutations in the neighboring genes between the mutant and the wild type. We also knocked down *GmPPD* genes and reproduced both the *mog* and *rl* phenotypes in soybean. Controlling *PPD* genes to produce the *mog* phenotype is highly valuable for breeding since larger seed size could directly increase the commercial values of grain legumes.

Key Words: seed size, map-based cloning, legume, blackgram, soybean, biomass.

Introduction

Seed size is one of the most important domestication-related traits for leguminous crops. Compared to the wild ancestors, most domesticated legumes have much larger seeds (Abbo *et al.* 2014). Larger seed size is still a major target trait for modern breeding, because it is related not only to yield but to commercial value. In some countries, large-seed cultivars of leguminous crops are traded at 2–3 times higher price than small-seed cultivars.

However, seed size is a typical quantitative trait, which is sum of multiple loci with small effects. Many genetic studies on seed size have been performed to date, but even the largest QTLs in those studies could increase the seed size by only 20% (Isemura *et al.* 2010, 2012, Kaga *et al.* 2008,

Kongjaimun *et al.* 2012, Zhang *et al.* 2004).

For blackgram (*Vigna mungo* (L.) Hepper), however, which is one of the most important legume crops in South Asia, a doubled seed size mutant was obtained by gamma-irradiation to a cultivar ‘Phitsanulok 2’ (used to be called as ‘BC 48’) (Chinchest and Nakeeraks 1990). The mutant plant also had larger leaves and stems, thus we designated it as *multiple organ gigantism (mog)* mutant. Although a genetic linkage map was constructed using a BC₁F₁ population derived from the *mog* mutant and a wild accession ‘TC2210’ (Chaitieng *et al.* 2006), no further analyses have been reported.

Here we evaluated growth characteristics of the *mog* mutant and demonstrated the *mog* mutant was not only bigger but grew faster than the wild type. We also performed a map-based cloning and successfully identified that a mutation in *VmPPD* gene in the *mog* mutant. We also knocked down the orthologous genes in soybean (*Glycine max*) and successfully reproduced the *mog* phenotype.

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Materials and Methods

Plant materials

Seeds of *V. mungo* (*mog* mutant (JP219132), 'Phitsanulok 2' (JP106710) and TC2210 (JP107873)) were provided by NARO genebank.

Evaluation of growth characteristics

To evaluate growth characteristics of the *mog* mutant and Phitsanulok 2, 16 plants per line were grown in plastic pots, from July to October in 2004, in a greenhouse with a 12 h dark/12 h light condition. Leaf area was measured by ImageJ (Schneider *et al.* 2012) after scanning. Number of cells/mm² were calculated by observing epidermis on the fifth leaves in 10 plants using a stereomicroscope. Cell numbers were counted in the microscopic field, which was estimated to be 0.14 mm². Four plants were sampled for measuring total leaf area and total dry weight of vegetative tissues (leaves and stems) on the 20th, 40th and 80th days after planting. Pods and seeds were harvested on the 90th day after planting.

Linkage analysis

The first linkage analysis was conducted using 191 F₂ plants derived from the *mog* mutant and TC2210. Plants were grown from July to October in 2005 in the same greenhouse described above. Seeds were harvested from each plant and were measured to calculate 100 seeds weight. DNA extraction, genotyping, linkage map construction and QTL analysis was performed as described by Kaga *et al.* (2008). Detailed information of the SSR markers are available from [https://www.gene.affrc.go.jp/databases-marker_information_en.php].

To develop a population for fine mapping, we selected 51 F₂ plants that harbored the marker loci CEDG156 and CEDG271 as heterozygous and as many other loci fixed with alleles of TC2210 as possible. We backcrossed TC2210 as the recurrent parent to the selected F₂ plants and obtained 466 BC₁F₁ seeds. Of the 466, we selected and cultivated 24 BC₁F₁ plants, performed the second backcross and obtained 519 BC₂F₁ seeds. We genotyped 200 of the 519, selected 32 and performed the third backcross. Of the 384 BC₃F₁ seeds obtained, we selected two plants, selfed them and obtained 1,564 BC₃F₂ seeds.

We cultivated the BC₃F₂ population in plastic pots in the greenhouse from May to August in 2008 with a condition of 12 h dark/12 h light. We evaluated each plant to confirm whether the leaves were rippled. Seeds were harvested from each plant and were measured for 100 seeds weight. To determine whether the BC₃F₂ plants were fixed with the wild type allele or heterozygous at the *MOG* locus, we sowed BC₃F₃ seeds of the 1,139 BC₃F₂ plants (50 seeds per line) whose leaves showed the wild type phenotype. We cultivated the plants in the field from June to August in 2009. We evaluated all the plants for the *rl* phenotype.

To develop more markers, we first BLASTed the marker

sequences of CEDG156 and CEDG271 in soybase v1.1 (<http://soybase.org/>) (Grant *et al.* 2010) to identify the syntenic region of the *mog* locus in soybean (*Glycine max* (L.) Merr.). We then downloaded the genome shotgun sequences of mungbean (*V. radiata* (L.) R. Wilczek) (Tangphatsornruang *et al.* 2009) and retrieved those aligned to the syntenic region of soybean. From the retrieved sequences, we designed 69 SSR markers using read2marker (Fukuoka *et al.* 2005) (**Supplemental Table 1**). Of the 69, nine were found polymorphic between the *mog* mutant and TC2210 and were used to genotype the BC₃F₂ plants.

Around the marker MBGS46949, eight genes were present in the soybean genome v1.1 (Schmutz *et al.* 2010). According to the genome shotgun sequences of mungbean (Tangphatsornruang *et al.* 2009), we designed primers to sequence introns of the eight genes in blackgram (**Supplemental Table 2**). Since we found no polymorphism except single nucleotide substitutions in all the eight genes between the mutant and TC2210, we designed CAPS (Konieczny and Ausubel 1993) or dCAPS (Neff *et al.* 1998) markers to distinguish the parental genotypes (**Supplemental Table 3**).

BAC library

After we found the four gene loci were completely linked to the *mog* phenotype, we screened the BAC library constructed from genomic DNA of the blackgram cultivar 'Phitsanulok 2'. The BAC library construction and screening were performed as described by Wang *et al.* (2015). The DNA markers "38930_set1" and "39010_set1" were used for screening (**Supplemental Table 2**). After screening, the BAC DNA of the clone VmR48p23 was extracted using ZR BAC DNA Miniprep Kit (Zymo Research). The extracted DNA was sheared into ~3 kb fragments, cloned into pUC118 vector, sequenced with BigDye Terminator (Thermo Fisher Scientific) and ABI3730 (Thermo Fisher Scientific), assembled by CLC Genomic Workbench (QIAGEN) and annotated manually. The sequence of the assembled BAC clone was deposited as LC101731 in DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>).

Sequencing candidate genes

We sequenced the whole ORFs of the four genes around the *MOG* locus in the *mog* mutant. Template DNA was amplified with KOD-plus (TOYOBO) and primers listed in **Supplemental Table 4**, and was sequenced with BigDye Terminator (Thermo Fisher Scientific) and ABI3730 (Thermo Fisher Scientific). The obtained sequences were aligned to the BAC sequence by CLC Genomic Workbench (QIAGEN).

Soybean transformation

To knockdown the *PPD* orthologues in soybean (*GmPPD1* and *GmPPD2*), we constructed a starting RNAi vector according to the following procedures. From the T-DNA destination vector pH7GWIWG2(I) (Karimi *et al.*

2002), we retrieved a DNA fragment containing the inverted Gateway and a 644-bp linker between the promoter and the terminator of the cauliflower mosaic virus (CaMV) 35S transcript by *SacI* and *KpnI* digestion. We then transferred the *SacI-KpnI* fragment into the *SacI-KpnI* sites of pUHG (Khalafalla *et al.* 2005), a plasmid vector for soybean transformation that contained a hygromycin phosphotransferase gene (*hpt*) and green fluorescent protein gene (*GFP*) as marker genes. The resulting vector was designated as pUHG:P35S-IR. For construction of the RNAi vector targeting *GmPPD* genes, an artificial 300-bp cDNA fragment was synthesized, cloned into pEX-A vector (Eurofin Genomics) and then transferred as described previously (Nishizawa *et al.* 2010) to pUHG:P35S-IR to produce pUHG:GmPPD-RNAi.

Transformation of soybean (cv. Jack) by particle bombardment and subsequent plant regeneration were performed as described previously (Khalafalla *et al.* 2005). The plants regenerated from somatic embryos tolerant to hygromycin B (Roche Diagnostics) were monitored with a fluorescence stereomicroscope (Leica), and those expressing the GFP were grown under the green house condition. A transgenic line transformed with the empty vector, pUHG (Khalafalla *et al.* 2005), was used as a control.

Quantitative Real-Time PCR

Total RNA was isolated from the young, unexpanded leaves of T₂ plants using RNeasy Plant Mini Kit (QIAGEN). One microgram of RNA was used to obtain cDNA with QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer's instructions. The amount of the transcripts was then quantified using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). All the qPCR amplifications were performed in quadruplicate in a total volume of 20 µl. Expression levels were measured using real-time RT-PCR and have been calculated using the delta-delta CT method (Livak and Schmittgen 2001) relative to the expression of *Tubulin* gene (Glyma.08g115100). The following primers were used for RT-PCR: *GmPPD1* 5'-CTGAGTAAGCTGGACTGGACTCGTT-3' and 5'-CTAGTGGATCATTTGGCAAATGAC-3, *GmPPD2* 5'-TTGAGTAACTGGACAGACTCACC-3' and 5'-CTAGTGGATCATTTGGGCAAA TGAC-3, *Tubulin* 5'-TCTTGGACAACGAAGCCATCT-3' and 5'-TGGTGAGGGACGAAATGATCT-3'.

Significance of transcriptional changes were tested by pairwise t-test with the Holm correction method (Holm 1979).

Results

To evaluate growth characteristics of the *mog* mutant, we grew the mutant and the cultivar 'Phitsanulok 2' (wild type) in a greenhouse and measured various traits several times until seeds were set (Table 1, Fig. 1).

Our survey revealed the mutant was bigger than the wild type in all organ sizes (Fig. 1A). Compared to the leaves in

Table 1. Summary of traits on seed production in 'Phitsanulok 2' and the *mog* mutant

	Phitsanulok 2	<i>mog</i>	Ratio	Significance ^a
Total seed weight (g)	18.6	17.9	0.96	n.s.
Number of seeds	397.5	226.5	0.57	**
100 seed weight (g)	4.7	7.9	1.69	**
Number of pods	62	47	0.76	*
Number of seeds/pod	6.4	4.8	0.75	*

^a***: Significant at 1% level; *: Significant at 5% level; n.s.: not significant, based on the analysis of variance.

the wild type, those in the mutant were 100% larger and were rippled (Fig. 1B, 1C). We also measured the area of the fifth leaf every two days and found that it took 21 days for the leaf to become fully expanded in both the wild type and the *mog* mutant (Fig. 1D).

To determine whether the *mog* phenotype was due to increases in cell numbers or cell sizes, we observed the leaf epidermises with a stereoscopic microscopy. As a result, the cell size was almost the same between the wild type and the mutant, indicating the gigantism was the result of increase in cell numbers (Fig. 1E).

Although the difference in the seed size was not so large as that in the leaf size, the *mog* mutant increased the seed weight by 70% compared to the wild type (Fig. 1F, Table 1). In addition, the *mog* mutant acquired systemic gigantism including total leaf area (Fig. 1G) and plant biomass (Fig. 1H), neither of which required longer time to reach plateau. The increase in these traits were also 50–70%.

As for the total weight of seeds, however, the increased seed size in the *mog* mutant was counterbalanced by reduced seed numbers, resulting in a similar total seed weight per plant to that of the wild type (Table 1). Compared to the wild type, the mutant produced 24% less number of pods and 25% less number of seeds per pod, leading to 44% less number of total seeds.

The traits of the mutant as described above provided us enough reason to map the mutant locus. Thus we developed an F₂ population by crossing an accession 'TC2210' to the *mog* mutant. TC2210 is an accession of wild blackgram and thus its seeds were much smaller (1.8 g/100 seeds) than the mutant or the wild type. After developing a genetic map (Supplemental Fig. 1), we measured 100 seeds weight of 191 F₂ plants and performed QTL analysis. The results revealed only two QTLs could explain more than 5% of phenotypic variance in seed size, and the one on the linkage group 8 (LG8) had an extremely strong effect that explained 65.9% of phenotypic variance with a LOD score of 66.9 (Table 2). We considered this locus, between the SSR loci of CEDG156 (22.7 cM) and CEDG271 (32.8 cM) on LG8, was the *MOG* locus (Fig. 2A).

To examine whether the *MOG* locus had been identified as seed size QTL in other legumes, we compared the blackgram linkage map with other Asian *Vignas* including azuki bean (*V. angularis* (Willd.) Ohwi & H. Ohashi) (Kaga *et al.* 2008), rice bean (*V. umbellata* (Thunb.) Ohwi & H. Ohashi)

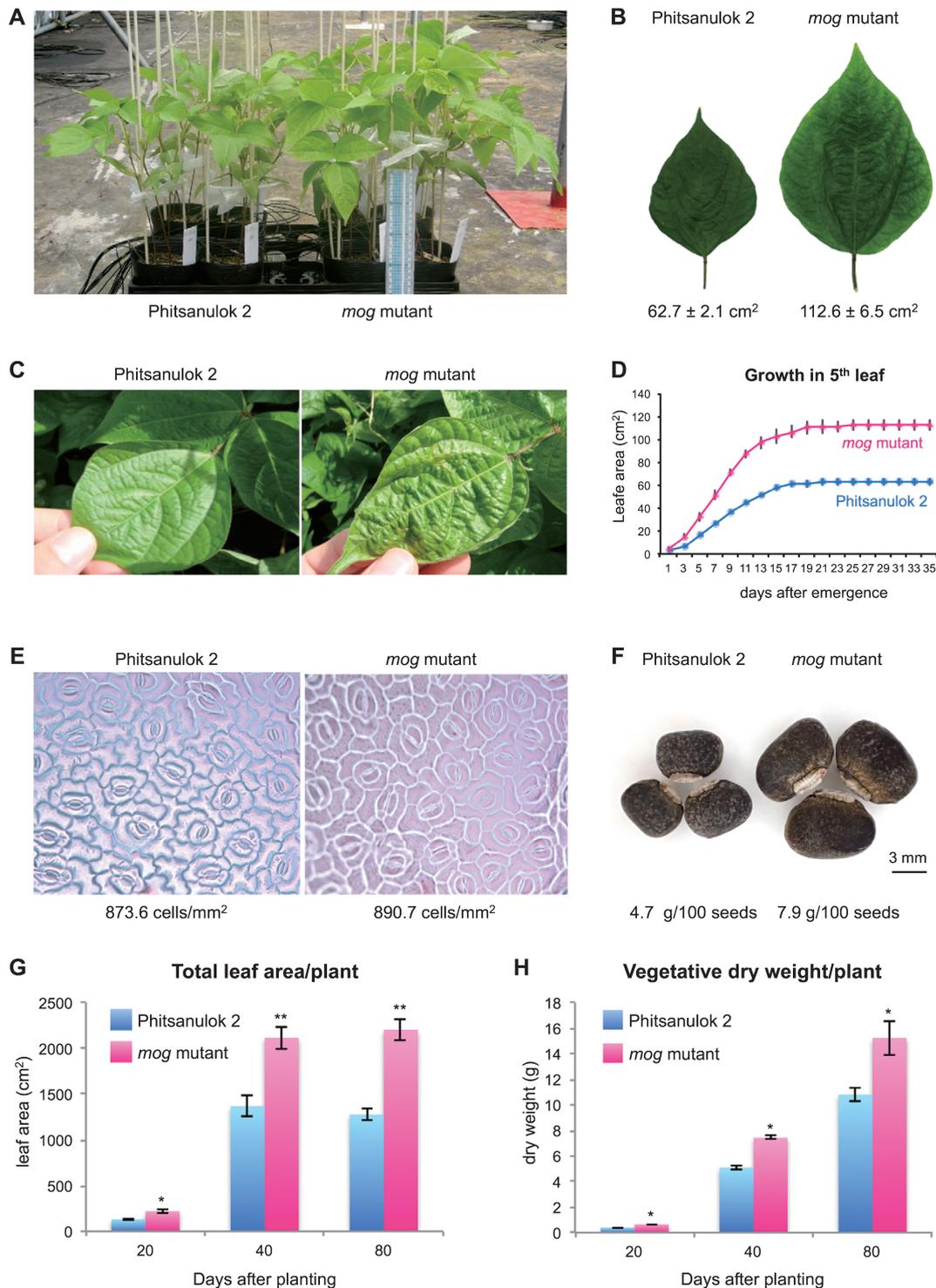


Fig. 1. Phenotypes of the wild type cv. ‘Phitsanulok 2’ and the *mog* mutant. (A) Whole plant. (B) The central leaflet of the fifth leaf. (C) The wild type leaf (left) and the rippled leaf in the mutant (right). (D) The growth of the fifth leaf after emergence. (E) Epidermal cells on the fifth leaf. (F) Seeds. (G) Total leaf area per plant after planting. (H) Total biomass per plant after planting. Significant differences between the wild type and the *mog* mutant are indicated by *: $p < 0.05$ and **: $p < 0.01$.

(Isemura *et al.* 2010) or mungbean (*V. radiata*) (Isemura *et al.* 2012). However, the *MOG* locus did not overlap with any seed size QTLs previously identified (**Supplemental Fig. 2**).

To finely map the *MOG* locus, we conducted three rounds of backcross to develop a BC₃F₂ population. Of the 1,564 BC₃F₂ plants, 425 showed the *rippled leaf* (*rl*) phenotype (see **Fig. 1C**) whereas the remaining 1,139 plants

Table 2. QTLs for 100 seeds weight detected in the F₂ population derived from the *mog* mutant and ‘TC2210’

LG	Position	LOD	PVE ^a	Additive effect	Dominance effect	Marker 1 ^b	Marker 2 ^b	P value
LG01	46.5 cM	4.9	2.0%	0.32 g	0.01 g	VES0025	VES0670	0.0005
LG02	40.4 cM	7.4	3.0%	0.38 g	0.14 g	VES0718	GMES1033	0.0001
LG03	6.8 cM	5.3	1.9%	0.34 g	0.01 g	cp00361	VES0120	0.0002
LG05	15.4 cM	4.9	2.0%	-0.33 g	-0.06 g	CEDG020	DMBSSR217	0.0001
LG07	42.5 cM	3.5	1.7%	0.30 g	-0.01 g	GMES5773	Bng152	0.0036
LG08	32.2 cM	66.9	65.9%	2.00 g	-0.51 g	CEDG156	CEDG271	0.0001
LG09	34.5 cM	13.6	5.6%	0.60 g	0.09 g	CEDGAG001	sgA060	0.0001
LG10	31.3 cM	7.8	3.0%	0.44 g	-0.04 g	cgP157	GMES4400	0.0001

^a Phenotypic variance explained.

^b Adjacent DNA markers.

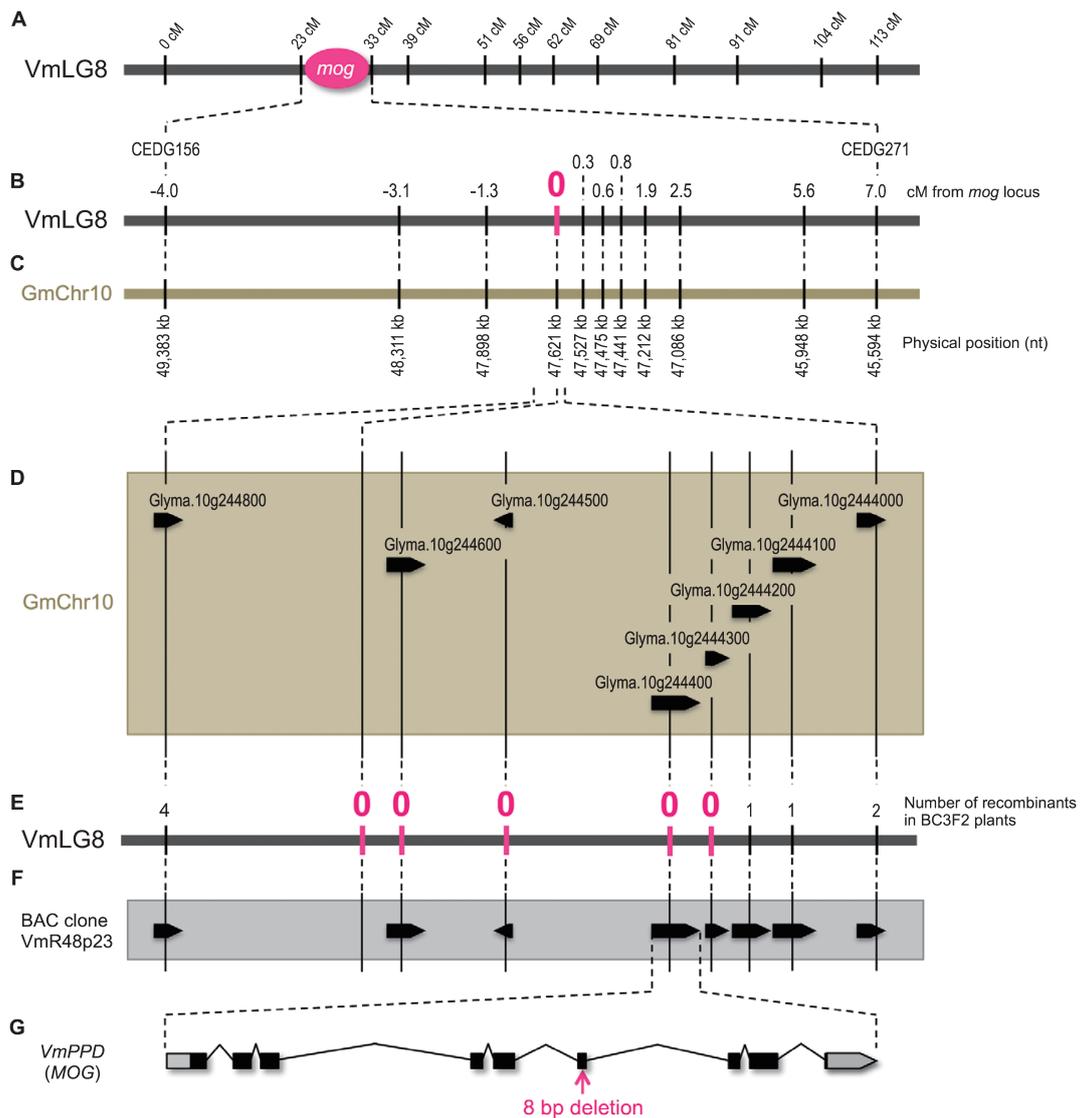


Fig. 2. Genetic and physical maps of blackgram and soybean around the *MOG* locus. The vertical solid lines indicate marker loci and the broken lines indicate corresponding positions between the maps. (A) The linkage map of blackgram LG8 (VmLG8). (B) A close up of the blackgram genetic map around the *MOG* locus. (C) A physical map of soybean chr10 (GmChr10), which is syntenic to the *MOG* locus. (D) A further close up of the soybean physical map around the marker locus MBGS46949, which was completely linked to the *MOG* locus. The black pentagons indicate genes. (E) A genetic map of blackgram syntenic to the soybean genomic regions shown in (D). (F) A physical map of the BAC clone VmR48p23. The black pentagons indicate predicted genes. (G) A schematic of *VmPPD* gene. The black and gray boxes indicate coding sequence and untranslated regions, respectively.

showed the wild type (*RL*) phenotype, suggesting the *rl* phenotype is controlled by a single recessive allele. In addition, all the *rl* plants produced larger seeds while others produced smaller seeds without any exception (2.9 g/100 seeds vs. 1.8 g/100 seeds, respectively) (**Supplemental Fig. 3**).

In addition, we cultivated the BC₃F₃ lines (50 plants per line) derived from the BC₃F₂ plants with the *RL* phenotype to determine whether the wild type plants have the genotype of [*RL/RL*] or [*RL/rl*]. Of the 1,139 lines, 756 lines generated plants with the *rl* phenotype while 383 lines generated only the *RL* phenotype. As such, the segregation of [*RL/RL*] : [*RL/rl*] : [*rl/rl*] was within the expected ratio of 1:2:1 ($X^2 = 3.98$, $p = 0.13$). Compared to the [*RL/RL*] plants, the heterozygous plants bore slightly larger seeds, further confirming both the *rl* and the *mog* phenotypes were controlled by the same allele in the *MOG* locus (**Supplemental Fig. 3**).

Since no genomic information of blackgram was available, we relied on the synteny between blackgram and soybean to obtain more DNA markers. The region containing the *mog* locus was syntenic to 49.4 Mb-45.6 Mb on chr10 in soybean and 36.9 Mb-41.4 Mb on chr20 (**Fig. 2A–2C**). Since both the soybean regions were totally syntenic to each other, we ignored the latter in this study. However, it was not easy to design new markers based on soybean sequence, because soybean and blackgram are genetically divergent. On the other hand, the genome shotgun sequences of mungbean, which belongs to the same taxonomic section of the genus *Vigna* as blackgram, were also available although the

assembly was highly poor (Tangphatsornruang *et al.* 2009). As such, we designed SSR markers based on mungbean sequences that were aligned well to the syntenic region of soybean. We obtained nine polymorphic markers, of which the locus MBGS46949 was completely linked to the *MOG* locus (**Fig. 2B**). In soybean, eight genes were present around this locus (**Fig. 2D**). Using the mungbean sequences, we designed primers to sequence introns of the blackgram orthologues and developed more DNA markers (**Fig. 2E**). Of the eight, we found four marker loci, four genes namely, were completely linked to the *MOG* locus (**Fig. 2E**).

However, it was still possible that this locus in blackgram contained other genes than the four. Thus, we constructed a genomic BAC library of the wild type blackgram and screened for a clone that contained the *MOG* locus. We identified VmR48p23 as such a clone, sequenced the whole clone and annotated. As a result, we identified only the four genes and found no other gene coding sequences in this locus (**Fig. 2F**).

We then sequenced the whole ORFs of the four genes in the *mog* mutant. As a result, we found no sequence alterations except an 8 bp deletion in the 6th exon of an orthologue of Glyma.10g244400 (**Fig. 2G**, **Supplemental Fig. 4**), which was also orthologous to *Arabidopsis* *PEAPOD* (*PPD*) gene (White 2006). Thus we designated the gene as *VmPPD*.

To further confirm that *VmPPD* is responsible for the *mog* phenotype, we knocked down the *PPD* orthologues in

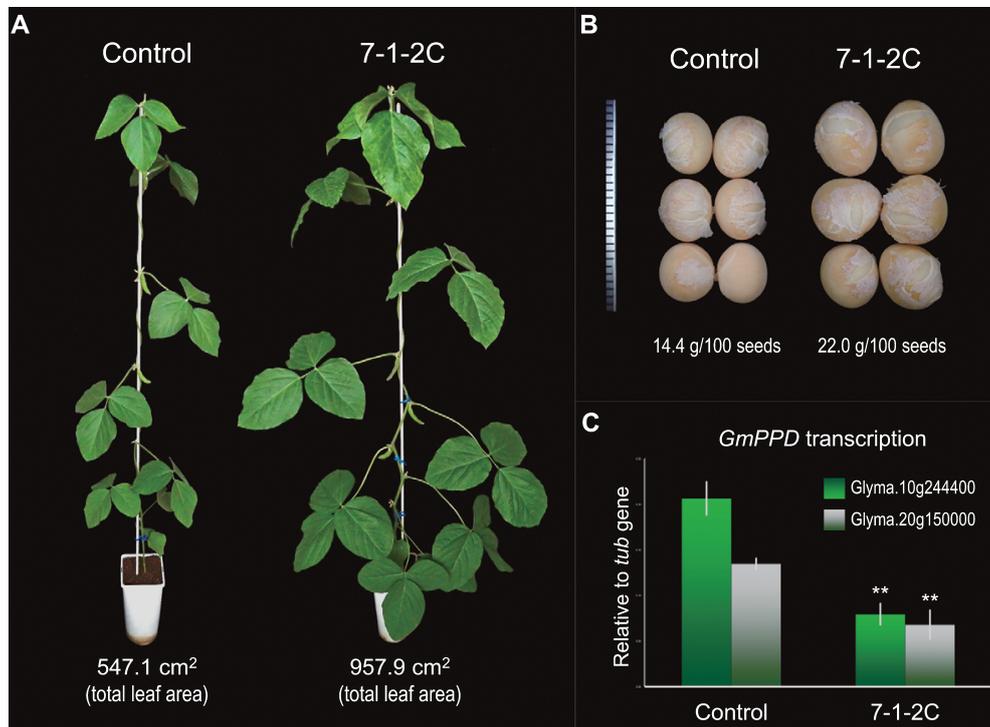


Fig. 3. The RNAi transgenic soybean for *GmPPD* genes and the control (empty vector). (A) The whole plant. (B) Seeds. (C) Transcriptional levels of *GmPPD* genes in young leaves. The error bars indicate standard deviation ($n = 4$). Significant differences between the control and the transgenic are indicated by *: $p < 0.05$ and **: $p < 0.01$.

Table 3. Summary of traits on seed production in transgenic soybean

	7-1-1B	7-1-2C	7-2-1E	Control
Total seed weight (g/plant)	4.4	5.6	2.2*	3.7
Number of seeds/plant	25.5	20.8	8.5**	26.5
Seed weight (g/100 seeds)	17.1*	22.0**	26.1**	14.4
Number of pods/plant	10.2	10.5	7.0*	11.3
Number of seeds/pod	2.6	2.0	1.2*	2.3

**Significant at 1% level.

*Significant at 5% level.

soybean (*GmPPD1*; Glyma.10g244400 and *GmPPD2*; Glyma.20g150000) by RNAi (Fig. 3, Supplemental Fig. 5). Of the six independently-transformed T₂ lines obtained, we found three lines (7-1-1B, 7-1-2C and 7-2-1E) were clearly bigger than others and had significantly suppressed the *GmPPD* transcriptions (Fig. 3C, Supplemental Fig. 6). Compared to the control (transgenic with empty vector), all three lines showed bigger leaves (Fig. 3A) and significantly bigger seeds (Fig. 3B, Table 3). The leaves of the RNAi plants were also rippled (Figs. 1C, 3A). The 7-1-2C plants produced higher yield (total seed weight) than the control plants, although the difference was not significant (Table 3). The 7-2-1E plants were even bigger than the 7-1-2C plants, but produced significantly less pods, less seeds and lower yield than the control plants (Table 3). The number of seeds per pod was also significantly lower in 7-2-1E than in control (Table 3).

Discussion

In this study, we demonstrated that the *mog* and *rl* phenotypes in the blackgram mutant was due to a loss-of-function mutation in *VmPPD* gene, an orthologue of *Arabidopsis* *PEAPOD* (White 2006). This mutation led to 100% increase in leaf size, 50–70% increase in biomass, and >70% increase in seed size (Figs. 1, 3). The increase of seed size by the mutation is by far the largest, compared to other seed size QTLs identified in *Vigna* crops, which increase seed size by no more than 20% (Isemura *et al.* 2010, 2012, Kaga *et al.* 2008, Kongjaimun *et al.* 2012, Zhang *et al.* 2004) (Supplemental Fig. 2). Although *ppd* mutants or *PPD*-knockdowns did not necessarily achieve higher yield of total seed weight (Tables 1, 3), the larger seed size could improve the commercial value of leguminous crops.

The function of *PPD* genes were originally elucidated in *A. thaliana*, where the null mutant of *AtPPD* genes showed increased leaf size (White 2006). Because *PPD* genes serve to arrest cell divisions in meristematic cells, malfunction of *PPDs* leads to more cell divisions and increased organ sizes (Fig. 1E). Although no change in seed size has been reported for *ppd* mutants or *PPD*-knockdowns in *Arabidopsis*, it might be because seed size is determined by cotyledons in legumes but by endosperms in *Arabidopsis* (Olsen 2004). As such, at least in legumes, *PPD* is also involved in seed size regulation. The recent report on a deletion mutation of *PPD* in *Medicago truncatula* also expresses the *mog* pheno-

type (Ge *et al.* 2016), further supporting our conclusion that the loss-of-function mutation in *VmPPD* is the cause for the *mog* phenotype.

We should mention here that *PPD* is one of the broadly conserved transcription factors in plants (Bai *et al.* 2011). Although the monocot species has lost this family, most of the dicot species, and even Lycopodiophyta species, have at least one copy of *PPD*. Since the function of *PPD* genes are conserved at least between legumes and Brassicaceae, we expect it is also conserved in other Dicots.

As such, it would be possible to increase leaf size and biomass by suppressing *PPDs* in horticultural crops. In addition, the faster growth by suppressing *PPD* (Fig. 1D, 1G, 1H) may accelerate harvesting cycles, especially for leafy vegetables cultivated in plant factories. As genome-editing via CRISPR/Cas system (Baltes and Voytas 2015) and virus-induced gene silencing (Ramegowda *et al.* 2014) are being practical in various agricultural and forestry species, the application of *PPD*-knockouts could be realized in the near future.

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